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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

Editor: Michael C. Venuti
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SPECIAL TOPIC

Chapter 29. Macromolecular X-Ray Crystallography and NMR as Tools for Structure-based Drug Design

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Introduction - The search for novel and patentable lead compounds is the hallmark of all drug discovery research efforts. How are these compounds discovered? Historically, most leads have been found by screening natural products and chemical libraries. However, over the past decade, advances in biotechnology have ushered in a new approach to drug discovery termed *structure-based drug design*. First envisioned by Abraham nearly 20 years ago (1) and pioneered by Goodford in the *de novo* design of antisickling agents that bind hemoglobin (2), structure-based design utilizes knowledge of the structure of a ligand complexed to its macromolecular target to design new lead compounds or to improve the potency and physical properties of an existing chemical series (3). In principle, all classes of macromolecules - proteins, nucleic acids, carbohydrates, and lipids - can serve as drug design targets. In practice, the experimental determination of macromolecular structure to atomic accuracy has been mainly applied to proteins or proteinaceous assemblies, such as protein-nucleic acid complexes or viral capsids. In this chapter we limit our discussion to protein targets with the understanding that many of the concepts and examples described herein may be generalized to other macromolecular systems of interest.

Despite much theoretical effort to fold protein structures based on their amino acid sequences, the determination of protein structure remains a largely experimental enterprise, and usually requires the application of macromolecular X-ray crystallography or NMR techniques. In certain cases where the three-dimensional structure of a homologue of the target enzyme or receptor is known experimentally, a plausible starting structure may be derived using homology modeling approaches (4). This strategy has been used extensively in the design of renin inhibitors (5). In any case, the key consideration for structure-based design is the generation of a reliable three-dimensional atomic model. Several comprehensive reviews provide earlier examples of the use of protein and small-molecule crystal structures in ligand design (2,3,6). This review will discuss the uses of X-ray crystallography and NMR, either alone or in combination, in the context of recent structure-based drug design scenarios.

X-RAY CRYSTALLOGRAPHY IN DRUG DESIGN

X-ray crystallography is a well-established technique that can produce atomic resolution structures of proteins and their complexes with various ligands such as substrates, co-factors or inhibitors (7). From a purely conceptual standpoint, protein crystal structures may be used to aid drug design in several distinct manners: *a posteriori* analysis, *a priori* design, and iterative design. *A posteriori* analysis is a retrospective method whereby protein structure is used to rationalize existing SAR data and to propose design improvements. This has probably been the most frequently used mode of protein crystallography in early ligand design efforts. *A priori* design refers to the use of protein structure for the design or discovery of the initial lead compound. This mode of design holds promise for the invention of structurally novel leads, and its usefulness will undoubtedly grow in conjunction with the development of computational approaches such as GRID (8), ALADDIN (9) and DOCK (10) that utilize protein structures to design or to search for complementary small

molecules that could serve as new leads. Iterative design refers to the situation where all elements of the drug design cycle are in dynamic feedback - structure determination, analysis, design, synthesis, and testing. From a practical standpoint, iterative design will entail both *a posteriori* and *a priori* design during the optimization process. In addition, iterative design requires the close coordination of an interdisciplinary team effort as well as a constant supply of target receptor for crystallization studies. For these reasons, iterative design has only recently been feasible using X-ray crystallography as an integral component.

Human Rhinovirus Inhibitors - *A Posteriori* Design - Human rhinoviruses cause the common cold, and over a hundred different isolates have been identified for this picornavirus. A novel class of oxazolinylphenyl isoxazole compounds (*e.g.*, 1-5) were demonstrated to possess broad spectrum antiviral activity against picornaviruses (11). The mechanism of action of these compounds was shown to be due to inhibition of the viral disassembly step of the infection process. The structural basis for this activity was demonstrated with the crystallographic structure determination of a complex of WIN 52084 (1) with the capsid of human rhinovirus 14 (HRV14) (12). The lipophilic drug is nestled snugly within a hydrophobic core between the two β-sheets that comprise the β-barrel of the VP1 coat protein subunit (Figure 1A). There is a 25 Å deep, continuous depression or "canyon" that surrounds the icosahedral vertices of the surface of HRV14 virions. The canyon is formed by the icosahedrally-related pentamers of VP1 subunits, and has been implicated in receptor binding (13). The entrance to the drug-binding site in VP1 can be visualized as a pore at the base of the canyon floor (Figure 1B). The isoxazole moiety of 1 is buried deep within the pocket, under the floor of the canyon, and the oxazolinyl end occupies a position near the pore. These results help to explain the slow association rate (k_{on} ~ 167,000 $M^{-1}min^{-1}$) of disoxaril (3) as being due to restricted access to the binding pocket and to a requirement to displace water molecules occupying the pocket in order for binding to occur (11). Antiviral studies demonstrated that the (S) isomer at the 4-position of the oxazoline ring was approximately ten-fold more potent than the (R) isomer. This stereoselectivity was mirrored in the preference of the (S) over the (R) isomer binding to HRV14 in crystal soaking experiments using racemic mixtures of 1 or analogs. Energy profile analysis suggested that the 4-methyl moiety in the (S) configuration is able to make more favorable hydrophobic interactions with the protein (11). X-ray crystallographic investigation of a series of structurally-related drug/virus complexes (14) revealed the occurrence of a second mode of binding in which the inhibitor is oriented in the pocket with the opposite directionality (Figures 1C and 2) . In this case, the isoxazole moiety is closest to the opening of the canyon. Interestingly, both orientations place a methyl group in roughly the same hydrophobic region near the side chain of Leu106. This may not be coincidence, since the presence of an alkyl moiety at the 4-position on the oxazoline moiety is apparently required for the tighter mode of binding in which the oxazoline ring is positioned near the pore (1(S) and 2(S)). Unsubstituted oxazolines (3 and 5) and compounds with shorter linkers (4) bind with the

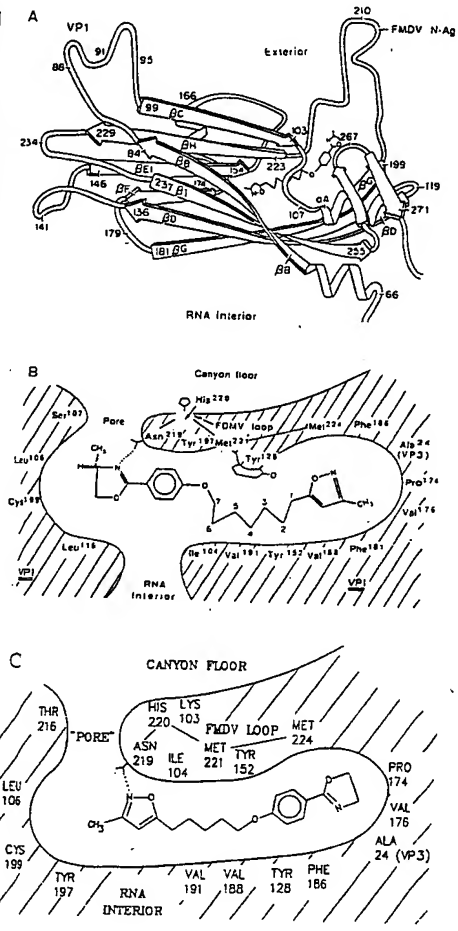


Figure 1. (A) Ribbon diagram of VP1 with WIN compound binding site. Diagrammatic representations of (B) compound 1 and (C) compound 5 bound in the WIN pocket. Reprinted with permission from the American Association for the Advancement of Science (12) and the National Academy of Sciences (14).

methylisoxazole nearer to the pore. Computational studies indicated the importance of space-filling, van der Waals interactions for the binding of this class of compounds (15).

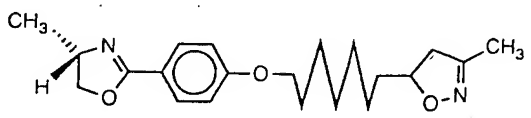
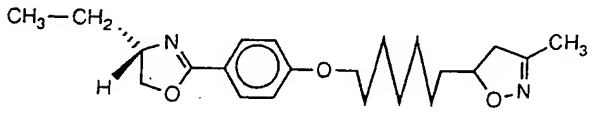
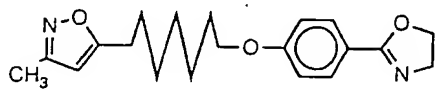
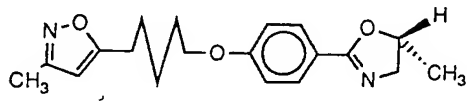
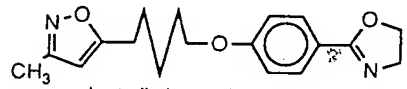
		MIC (μM)
1 (S)		0.03
2 (S)		0.02
3		0.6
4 (S)		0.6
5		0.5

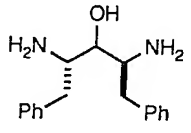
Figure 2. Some of the compounds studied crystallographically when complexed to HRV14. The relative orientations of the bound compounds are illustrated with respect to the "pore" to the canyon floor which is on the left. Adapted from (14), with permission of the National Academy of Sciences.

In addition to providing a structural basis for the antiviral effects and relative potencies of 1 - 5, the crystallographic studies indicated that drug binding induced substantial structural changes in VP1 causing main chain atom movements up to 4.5 Å and shifts of up to 7.5 Å in the positions of side chain atoms (14). These changes near the drug binding site also caused conformational changes to the viral surface that resulted in a shallower canyon. These observations prompted speculation that drug binding might also inhibit virion attachment to the cell surface receptor. Binding studies using radiolabeled virus and cell membranes indicated that this was indeed the case (11,16). However, the cell attachment activity of the isoxazole compounds is not essential for antiviral activity since some strains of rhinovirus, such as HRV1A, that are inhibited by these compounds are unaffected in this step (17). X-ray crystallographic analysis of the HRV1A capsid structure indicated that the wild-type virus had a canyon conformation similar to that of the drug-bound form of HRV14 (17). Presumably, the antiviral compounds in this series exert their inhibitory effects through stabilization of the coat protein. The loss of flexibility accompanied by drug binding to the interior of VP1 results in the inhibition of disassembly. These structural studies have led to new insights into the mechanism of antiviral inhibition for 1 - 5 and can be used not only to assist the optimization of inhibitor potency for this class of compounds, but also to suggest new strategies for *de novo* design of antiviral agents against various strains of HRV as well as other picornaviruses. More generally, this example suggests a rationale that could be extended to the design of inhibitors of any macromolecular target whose function depends upon the flexibility of β-barrel-like structures.

HIV Protease Inhibitors - *A Priori* Design - The human immunodeficiency virus encodes a protease (HIV PR) that is essential for viral replication. HIV PR has been the subject of intensive drug design efforts and there are a number of recent excellent reviews on HIV PR structure (18) and inhibitor

design (19,20). Most of the medicinal chemistry approaches employed to design HIV PR inhibitors have been based on classical substrate or transition-state analog based approaches (21). However, three recent examples emphasized structural considerations in the *a priori* design or discovery of novel lead compounds.

The first example utilized the concept of active site symmetry to design two-fold (C_2) symmetric or quasi-C_2 symmetric inhibitors (22,23). The X-ray crystal structure of the protease of Rous sarcoma virus (24) revealed that retroviral proteins are homodimeric enzymes and that they are structurally-related to the aspartic proteinases, or pepsins (25). The suggestion that the active site of HIV PR was C_2 symmetric led to the design of simple lead structures that would mimic this symmetry and also satisfy the hydrogen bonding and subsite preferences known to be important for binding based on structural studies of other aspartic proteinase/inhibitor complexes (25). For productive binding of a symmeric inhibitor to occur, it would be necessary for the C_2 axes of the enzyme and inhibitor to approximately superpose in the complex.



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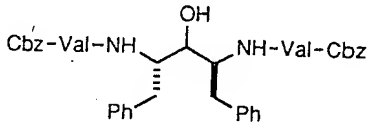

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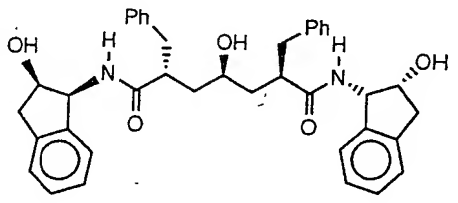
Modeling studies using the structures of RSV protease (24) and a complex of a reduced peptide renin inhibitor bound to rhizopuspepsin (26), a structurally related aspartic proteinase, indicated that 6 (IC_{50} > 100 mM) should be able to bind in the predicted manner. Elaboration of 6 by symmetrical addition of Cbz-blocked valine residues to enhance interactions with the P2 and P2' subsites in the enzyme resulted in a much more potent inhibitor, A-74704 (7; IC_{50} = 3 nM). X-ray crystallographic analysis of the structure of a complex of 7 bound to recombinant HIV PR verified that this pseudo-C_2 symmetric inhibitor bound in a highly symmetric fashion (22). This strategy has been used to design other classes of C_2 symmetric inhibitors that contain a central diol (23) or a difluoroketone (27). The C_2 or quasi-C_2 symmetric diols and difluoroketones are generally at least 10-fold more potent than their respective monohydroxy homologues. More recently, L-400,417 (8), a quasi-C_2 symmetric hydroxyethylene - based inhibitor of HIV protease has been reported (28). This compound differs from previous designs in that it is based on a C-terminal, rather than an N-terminal, duplication and thus contains an opposite directionality of the hydrogen bonding amides. X-ray crystallographic analysis of a complex of 8 with HIV PR revealed that the inhibitor bound in a nearly symmetric manner and formed many of the same backbone-backbone hydrogen bonds observed with 7, with the exception of the 2-hydroxy group on the indan which was hydrogen-bonded to the carbonyl oxygen of Gly27 and the amide nitrogen of Asp 29. The high potency of 8 (Ki = 0.67 nM) may be due to the incorporation of the novel 2-hydroxy-1-aminoindan moiety in P2' which was previously shown to strongly enhance binding in a classical hydroxyethylene peptidomimetic series (29). The above examples illustrate how knowledge of enzyme structure was used for the conceptualization of novel lead compounds. The lack of sufficient quantities of recombinant enzyme combined with an unusually rapid development of sub-nanomolar inhibitors in several C_2 symmetric classes precluded an iterative drug design approach in the first case. However, the structure of the A-74704/protease inhibitor complex was used to guide the design of pharmacological improvements by focusing synthetic efforts on the peripheral N-terminal blocking groups. Substitution of a 2-pyridyl-N-methyl urea for the Cbz-carbamate in the C_2 symmetric diol series led to 9 (enzyme IC_{50} = 0.15 nM; viral IC_{50} = 0.03-0.27



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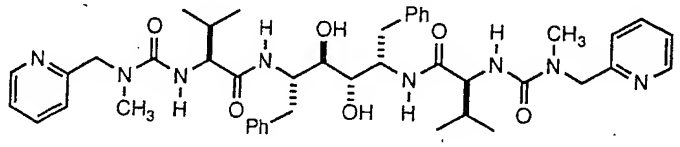

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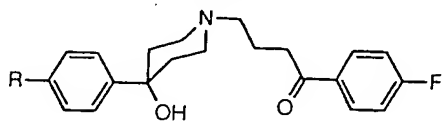
μM) which was moderately soluble (197 μg/ml) and exhibited achievable antiviral blood levels in monkeys (Cmax = 0.16 μM; Tmax = 1.5 hr) and dogs (Cmax = 0.59 μM; Tmax = 0.17 hr) upon oral administration (30).

In the second example of *a priori* lead compound discovery, the HIV PR structure was used directly to search structural databases for compounds that contained shapes complementary to the enzyme active site (31). The search procedure employed the program DOCK (10) which was developed to identify accessible surfaces or cavities on macromolecules and to convert these into "negative" images that can be used to search a structural database that has been converted into a corresponding set of surface representations. Using this technique, the known compound bromperidol (10) was identified and a closely-related analogue, haloperidol (11), was shown to weakly inhibit the enzyme (Ki=100 μM) (31). Bromperidol was originally discovered and modeled into the enzyme active site using the structure of the native enzyme to construct the search image. This result was rather surprising as it was later determined that the surface structure of the active site is dramatically altered upon inhibitor binding. The twofold-related flaps undergo a topological rearrangement and movement by up to 7-10 Å in order to engage a peptide-based inhibitor (32).



10 R = Br
11 R = Cl

In a third example of a structure-based search for new leads, the crystal structure of the A-74704/HIV PR complex was used to construct pharmacophoric targets that mimicked essential features of the inhibitor-enzyme interactions (33). Key pharmacophores were the central OH bound to the active site aspartic acids, the flanking amides bound to the main chain carbonyl oxygen atoms of Gly21 and Gly121, the buried water that bridges the flaps with the inhibitor, and the bulky phenyl group that binds in the P1 pocket. The corresponding pharmacophore search targets contained a central OH, two hydrogen bond donors, a hydrogen bond acceptor, and an aromatic moiety, respectively (Figure 3). Various combinations of these pharmacophores were used to search several chemical databases of three-dimensional structures for structurally novel compounds that might display inhibitory activity against HIV PR. A series of dibenzophenones was identified by this analysis. Several compounds were tested and shown to be inhibitory in the 10-100 μM range (Figure 4; 12-14). Structural considerations based on preliminary modeling studies indicated that the bulky R1 group on these compounds would make unfavorable van der Waals contacts with flap residues in the inhibited structure of HIV PR. Thus, either the flaps must maintain a more open conformation in complexes with these inhibitors, or the inhibitors might bind in a different orientation from that based on the model. It is interesting to note that statine-based peptidomimetics that contain an unsubstituted *p*-benzoylphenylalanyl moiety in the P1 position can be potent inhibitors of aspartic proteinases (34).

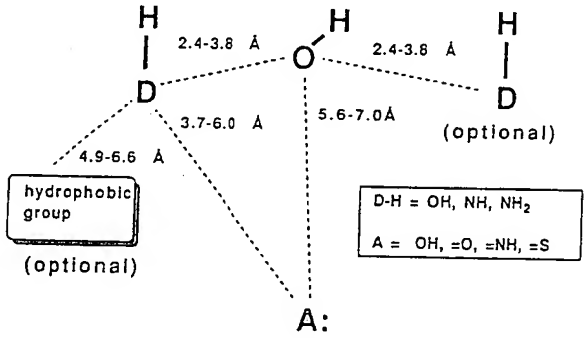


Figure 3. Schematic representation of pharmacophoric substructures used to search for non-peptidic inhibitors of HIV PR based upon the X-ray crystal structure analysis (22). D and A indicate hydrogen bond donors and acceptors, respectively. From (33), with permission from Tetrahedron Comp. Meth.

Iterative Drug Design - The convergence of rapid advances in biotechnology, particularly in the areas of recombinant gene expression and protein crystallography, has finally made it feasible to engage in the drug design cycle in an iterative and timely fashion. Two recent examples deal with the design of inhibitors for thymidylate synthase (TS) (35) and purine nucleoside phosphorylase (PNP) (36). In the TS example, the structure of the homologous enzyme from *E. coli* was used as a surrogate target for the design of human TS inhibitors. TS catalyzes the conversion of deoxyuridylate to thymidylate (dTMP) *via* an unusual, one-carbon transfer, methylation reaction that

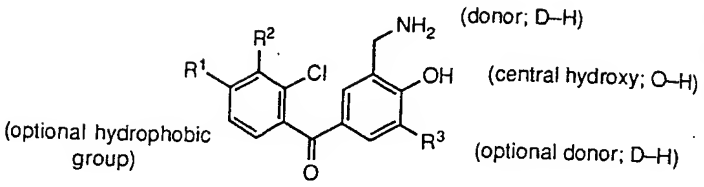
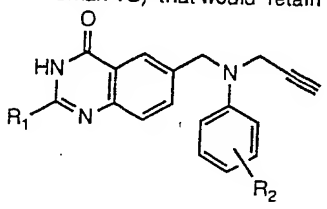


Figure 4. Non-peptide HIV PR inhibitors identified from the ALADDIN substructure search. Shown in parenthesis are the pharmacophores used in the search. IC_{50} values are for HIV PR inhibition at pH 4.5. Adapted from (33), with permission from Tetrahedron Comp. Meth.

	R^1	R^2	R^3	IC_{50}, μM
12	OCH_2COOEt	Cl	CH_2NH_2	11
13	OCH_2COOH	Cl	CH_2NH_2	85
14	OCH_2COOEt	H	Cl	15

utilizes 5,10-methylenetetrahydrofolate as a co-factor. TS comprises the sole and rate-limiting pathway for dTMP biosynthesis, and has been an important target for the design of antifolate antitumor agents that promise to exhibit superior activity to the clinically established dihydrofolate reductase inhibitor, methotrexate. Previous studies on the structure determination of TS from *L. casei* established the dimeric structure of the enzyme, and sequence comparison analysis suggested that the active site regions of homologous TS enzymes were highly conserved (37). A high expression recombinant system was developed for the *E. coli* enzyme, and led to X-ray crystal structure determinations for both the apo and ternary complex forms of TS (38-41). Upon analysis of these structures, it was evident that approximately 75% of the active site residues should be identical for the *E. coli* and human enzymes. Two different approaches were used for inhibitor design using the structure of *E. coli* TS: structure-based elaboration of an existing lead compound, and *de novo* design.

In the first approach, the goal was to develop a more lipophilic analog of the classical antifolate TS inhibitors, CB3717 (15) and 16 (Ki = 10 and 8 nM, respectively, for human TS) that would retain potency. Compounds related to 15 and 16 that contain the glutamate moiety are actively transported into cells and are thus subject to selective drug resistance. Removal of the *p*-CO-glutamate moiety from 16 led to a loss of 2.4 orders of magnitude in binding (17 ; Ki = 2.2 μM). Examination of the crystal structure of a complex of TS bound to 15, the 2-amino analogue of 16, indicated the presence of a small hydrophobic pocket in the enzyme proximal to the meta position of the phenyl ring. Compound 18 was modeled and synthesized and exhibited improved inhibition (Ki = 0.39 μM). The structure determination of 18 complexed with TS verified that the *m*-CF3 moiety occupied the modeled position and made van der Waals contacts with hydrophobic side chains of the enzyme. It was further predicted on electrostatic and structural grounds that a bulky hydrophobic group combined with an electron-withdrawing substituent positioned off the para position of the phenyl ring would further enhance inhibitor binding. This led to the modeling and synthesis of 19 which was nearly as potent as the lead compound 16 (Ki = 13 nM).



15 $R_1 = NH_2$, $R_2 = p$-COGlu

16 $R_1 = CH_3$, $R_2 = p$-COGlu

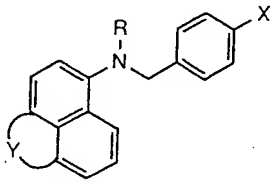
17 $R_1 = CH_3$, $R_2 = H$

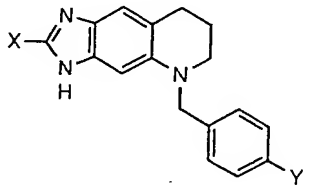
18 $R_1 = CH_3$, $R_2 = m$-CF_3

19 $R_1 = CH_3$, $R_2 = p$-SO_2Ph

In the second study, the aim was to use the inhibited form of the enzyme structure to design novel lead compounds *de novo*. The criteria for a working lead was that the compound exhibit a Ki below 10 μM, provide a crystalline complex with TS, and have a number of positions suitable for synthetic elaboration. Two crucial considerations were pointed out that are generically applicable to *de novo* design. First, the structure of the protein active site used for modeling should be that based on an inhibitor or a cofactor-bound form. This will ensure that any structural changes in the enzyme that are naturally induced by substrate or inhibitor binding will be reflected in the structure of the model. Secondly, the design of initial leads should build in soluble, polar groups . This will help to ensure that the initial, weakly potent leads will have the necessary solubility to be able to achieve concentrations in solution required for binding to the high concentrations of protein used in crystallization experiments. Two series of novel lead compounds were developed. The first series

evolved from the use of the program GRID (8) to predict the interaction energies between various functional group probes and enzyme atoms in the vicinity of the pteridine binding site. Based on this analysis and on an examination of the TS structure, compound **20** was modeled and synthesized (Ki = 3 μM). The key predictions leading to **20** were a hydrophobic pocket that could accommodate a naphthalene, a well-ordered water molecule in the crystal structure that could hydrogen bond to the carbonyl group at position 1, and the carboxylate side chain of Asp169 that would hydrogen bond to the NH at position 8. The latter two interactions could be made by bridging the 1- and 8- positions with a γ-lactam. Crystallographic analysis of a complex of **20** bound to TS revealed strategies for design improvements, and further iterative cycles of modeling, synthesis, X-ray crystallographic and computational energy analysis led to the design and synthesis of **21** with a Ki of 31 nM for human TS.





20 R = C_2H_5, X = SO_2–N⟨ ⟩NH_2^+ Cl^-, Y = 

22 X = H, Y = SO_2–N⟨ ⟩NH

21 R = CH_3, X = SO_2Ph, Y = 

23 X = NH_2, Y = SO_2–N⟨ ⟩NH

The second class of lead compound featured an imidazole moiety based on a detailed analysis of the hydrogen-bonding requirements of residues buried deep within the active site region. Hydrophobic and previous design considerations led to the elaboration of the substituted tetrahydroquinoline **22** (Ki = 7.7 μM). Crystallographic analysis indicated that **22** bound essentially as predicted. The feasibility of additional hydrogen bonding to a protein backbone carbonyl oxygen indicated the addition of an amino group at the 2-position of the imidazole ring that led to a 120-fold enhancement in potency (**23**; Ki = 64 nM). The 2-amino groups of **23** and of the classical quinazoline series, exemplified by **15**, occupied nearly identical positions in their respective complexes, and led to substantial binding improvements in both cases. Further cycles of iterative design based on **21** and **23** have led to more potent inhibitors for both classes of TS inhibitors (42,43).

A second recent example of iterative design (36) concerns the enzyme purine nucleoside phosphorylase (PNP) which participates in the purine salvage pathway and catalyzes the reversible phosphorolysis of purine ribo- or 2'-deoxyribonucleosides to the purine and ribose- or 2'-deoxyribose-α-1-phosphate. Inhibitors of PNP tend to exhibit T-cell toxicity and may have therapeutic potential in T-cell-mediated autoimmune disorders, T-cell leukemias and lymphomas, and organ transplantation. In addition, since PNP rapidly metabolizes purine nucleosides, inhibitors of this enzyme may be beneficial when co-administered with chemotherapeutic purine nucleoside analogs, such as the anti-AIDS drug, ddI. Human PNP is specific for 6-oxypurine compounds and is a trimer of identical subunits (total molecular mass = 97 kDa). Analysis of the structural differences between the apoenzyme and a complex with guanine revealed the existence of a "swinging gate" that moves up to several Å during substrate or inhibitor binding. This gate is formed by several amino acid residues of PNP and its complex motion could not have been predicted from the apoenzyme structure alone. Hence, the most fruitful modeling attempts were done with reference to the structure of the bound form of PNP, again emphasizing the importance for drug design studies of having the atomic coordinates for the receptor complex, and not just for the native receptor target. In the PNP example, X-ray structures were determined for complexes with a number of previously synthesized PNP inhibitors with micromolar Ki's, including 8-aminoguanine, 9-benzyl-8-aminoguanine, 5'-iodo-9-deazainosine, acyclovir diphosphate, and 8-amino-9-(2-thienylmethyl)guanine (36). The X-ray studies revealed several key features of inhibitor-enzyme interactions: i) 8-amino substituents enhanced binding of guanine analogs by forming hydrogen

bonds to Thr242; ii) 9-deaza guanine analogs enhanced potency through donation of a hydrogen bond to Asn243, and iii) addition of a bulky hydrophobic group at the 9-position of the purine improved binding *via* hydrophobic interactions with the ribose binding site. Based on this analysis, a number of 9-substituted 9-deazapurine analogs were modeled and synthesized (Figure 5). As expected, both 8-aminoguanine 9-substituted analogs and 9-deazaguanine analogs were potent inhibitors. Surprisingly, a combination of these features in the 8-amino-9-deazaguanine analog resulted in a relatively poor inhibitor. Analysis of 3.2 Å difference Fourier electron density maps from several of the relevant complexes revealed that two different modes of hydrogen bonding could occur with the side chains of Thr242 and Asn243 depending upon the protonation state of N-7 on the purine (Figure 5). This is a striking illustration of why it is important to experimentally determine structures of outliers during the iterative design process. The most potent compounds were those that were modeled based on a three subsite hypothesis: a purine subsite, a hydrophobic ribose subsite, and a phosphate binding site. The series of 9-deazaguanine 9-branched, benzyl-containing compounds (represented by 24) were stated to be the most potent membrane-permeable inhibitors of PNP reported thus far.

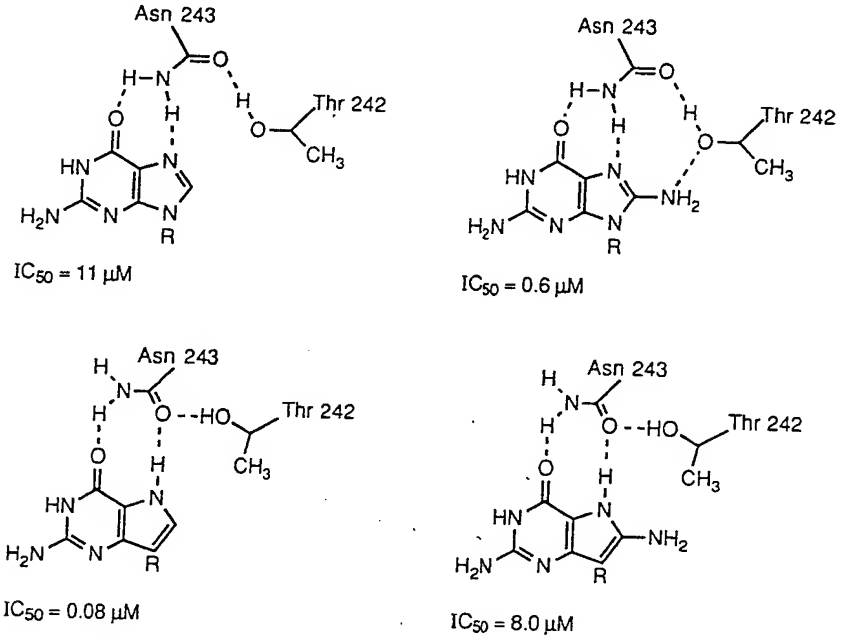
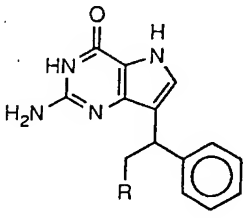


Figure 5. Comparison of hydrogen bonding interactions between 8-amino and 9-deaza modified purines with PNP based on X-ray crystallographic difference Fourier analysis. The R group is 2-thienylmethyl in the 8-amino purines, and 3-thienylmethyl in the 9-deazapurines. From (36), with permission from the National Academy of Sciences.

Besides the fact that structure-based design succeeded in helping to achieve nearly two orders of magnitude increase in inhibitor binding, several interesting considerations were borne out by this study. The difference Fourier maps extended to only 3.2 Å resolution - much less than the resolution that some would consider essential for drug design - and yet they proved to be exceedingly useful. The authors noted that over a 2.5 year period approximately thirty-five PNP-inhibitor complexes were evaluated using X-ray crystallographic methods, and roughly 60 active compounds were synthesized. This number of compounds is easily a tenth of the number that could be synthesized by a large medicinal chemistry team over the same period of time. Thus, the iterative drug design process could be viewed in utilitarian terms as having saved a great deal of synthetic effort that was productively utilized on other therapeutic projects. Finally, the X-ray crystallographic analysis was only able to keep pace with the modeling and synthetic efforts - a key aspect of iterative design - owing to the fact that the native PNP crystals could be readily soaked with



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PNP inhibitors. This was also true for the TS inhibitor iterative design example. In cases where soaking is not feasible, it is essential that co-crystallization conditions are found that permit the rapid growth (within one or two weeks) of high quality protein/inhibitor complex crystals.

Limitations and Future Developments - Of the essential steps required in a crystallographic structure determination - crystallization, X-ray diffraction data collection, data processing and reduction, phase determination, electron density map interpretation, and structure refinement - the serendipitous process of protein crystallization remains the major limitation in protein crystallography. Recent developments in crystallization robotics have improved the situation somewhat by enabling the use of large scale screening formats (44). Experience has shown that the more pure the protein is, the better are the chances of obtaining high quality crystals. The amounts of protein needed for a complete structure determination study vary from less than a mg to 100's of mg. The actual quantity required for a particular protein depends to a large extent on how rapidly one can identify reproducible conditions for crystal growth. Using microcrystallization techniques (44,45), a mg of protein can provide for numerous crystallization trials depending upon the desired protein concentration. For iterative crystallization studies, the required amount per structure drops dramatically owing to the fact that screening is no longer necessary, and in many cases individual crystals can be soaked with inhibitor. For *de novo* design, lead compounds should be designed to have aqueous solubilities high enough to allow for stoichiometric binding to the high protein concentrations (up to 1 mM) required for crystallization.

Phase determination for the indexed intensities, the second major hurdle in a structure determination, is done using one of several methods: molecular replacement, isomorphous (heavy atom) replacement, and anomalous dispersion. Isomorphous replacement requires measurements from one or more heavy atom-derivatized crystals and, thus, usually requires more material. The use of molecular replacement for phasing is restricted to proteins whose overall fold is suspected to be homologous to a protein of known structure. Recent advances in the direct phasing of native crystal structure amplitudes using multiple wavelength anomalous dispersion (MAD) has made it generally possible, in principle, to determine protein crystal structures without the need to resort to heavy atom substitution (46). This method avoids the unpredictability of derivatization and the lack of isomorphism that accompanies heavy atom replacement techniques. MAD uses the signal due to anomalous scatterers, like the sulfur atom of Cys or Met, that may be intrinsic to the protein, or that may be engineered into the protein by substituting selenomethionine for methionine, for example. The combination of powerful, tunable X-ray synchrotron light sources with recombinant technologies for producing anomalously-edited proteins promises an expansion of the use of MAD and accelerated phase determination for this crucial step in protein crystal structure determination.

The recent development of sensitive, high speed area detectors for rapid X-ray data collection and advances in affordable high speed computer graphics workstations are reducing the turnaround time for iterative structure determinations of drug/protein complexes to a few weeks or less. The point has been reached where structure analysis is becoming a rate-limiting step in the drug design cycle; *i.e.*, - structures are being solved more rapidly than one can perhaps effectively utilize them. The major challenge that lies ahead is clearly computational - to improve drug binding predictions both qualitatively as well as quantitatively in the search for more potent and effective cures for disease.

NMR IN DRUG DESIGN

Besides X-ray crystallography, nuclear magnetic resonance is the only other experimental technique that can provide structural details at atomic resolution. Since high quality crystals which may be difficult to obtain are not required, data collection may begin at an early stage in the structure determination. In addition, unlike with X-ray crystallography, NMR can continuously provide useful structural information on the way to determining the complete three-dimensional structure. These features and the fact that the NMR studies are conducted under different experimental conditions (in solution vs. in the crystalline state) suggest that NMR could also be a useful tool for designing new pharmaceutical agents (47,48). Indeed, isotope-aided NMR experiments have recently been developed for determining the conformation of a drug molecule when bound to its macromolecular receptor and for identifying those portions of the ligand that interact with the target site (49-54). This information could help distinguish between those functional groups of the ligand that are important for binding to the receptor from those that could be modified without affecting binding affinity, and could be used to design active analogs with better physical properties. This information could also prove useful for designing analogs with different molecular frameworks that would

position crucial functional groups of the ligand in their experimentally determined, spatial orientation.. These new analogs could have the advantage of being metabolically more stable or easier to synthesize (47,48).

In addition to those NMR studies that focus on the structure of the ligand and its structural environment, NMR could also be used to determine the complete three-dimensional solution structure of a drug/receptor complex. To obtain this structural information by NMR was impossible only a few years ago. However, due to rapid advances in heteronuclear three- and four-dimensional NMR techniques (55-62) and to the availability of isotopically labeled proteins (63-65), three-dimensional structures of medium-sized proteins (15-30 KDa) and molecular complexes can now be determined by NMR in a reasonable amount of time with good precision (66,67). The structural information that can be obtained from these studies, similar to that obtained from X-ray crystal structures, is important for determining the functional groups of the receptor responsible for ligand binding and for identifying new areas of the target molecule that may interact with suitably modified analogs. In general, the same approaches described in the previous section for designing new molecules based on X-ray structures could also utilize NMR-derived structures. Since high resolution structures have only recently been determined by NMR, the application of this technique is only in its infancy and has not been extensively appied to drug design. However, drug design based on NMR-derived structures may become more common due to recent advances which markedly extend the capabilities of this technique.

Advances in Multi-dimensional NMR Methods - One of the most important factors leading to the dramatic improvement in both the size and quality of the structures determined by NMR is the development of heteronuclear 3D and 4D NMR techniques (55-62). In early versions of these experiments (55), a heteronuclear shift correlation and a homonuclear 2D NMR experiment (*e.g.*, COSY, NOESY) were combined in such a way that homonuclear 2D NMR spectra are edited by the heteronuclear chemical shifts.. Thus, for large isotopically labeled molecules, proton NMR spectra can be resolved into many subspectra and rendered interpretable. A further increase in resolution can be achieved in a 4D NMR experiment by using another heteronuclear frequency (60-62).

Figure 6 illustrates the utility of ^{13}C-resolved 3D and 4D NOE experiments. In a 2D NOE experiment, NOEs between overlapping protons Ha/Hb and Hc/Hd cannot be unambiguously identified. By editing in f_1 using the ^{13}C chemical shifts in a 3D NOE experiment, the NOEs can be resolved in different planes. For example, NOEs involving Ha can be distinguished from those involving Hb by their appearance on different planes corresponding to the different frequencies of their attached carbons (Ca, Cb). However, the NOE crosspeaks in f_2 are only defined by their proton frequencies. Thus, the NOE between Ha and Hc or Hd appearing on plane Ca cannot be uniquely defined. By further editing of the proton signals in f_2 in a 4D NOE experiment, the NOE between Ha and Hc is unambiguously identified, since it appears on plane Cc and not Cd.

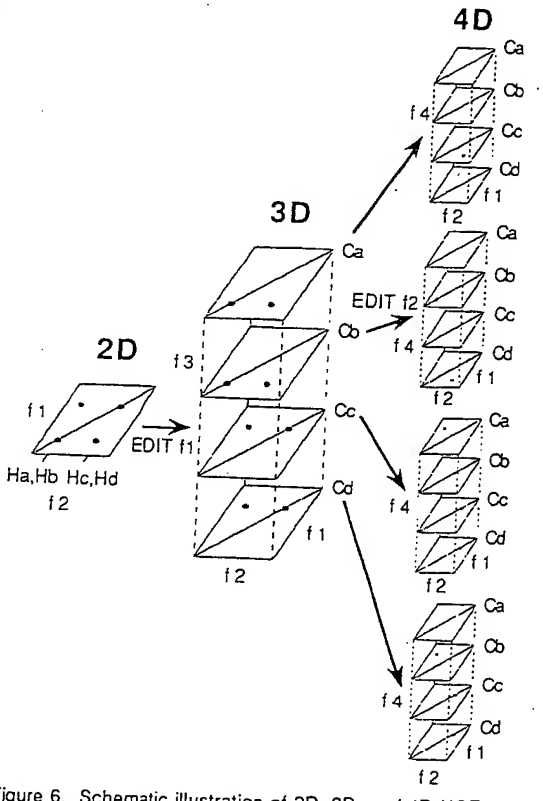


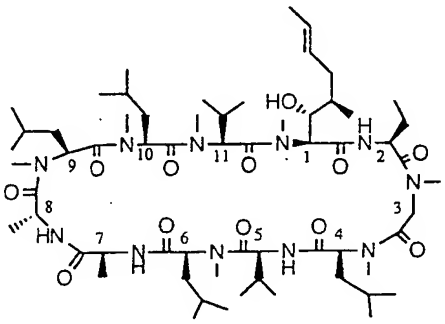
Figure 6. Schematic illustration of 2D, 3D, and 4D NOE spectra of protons with NOEs Ha/Hc, Hb/Hd, and Hc/Hd. From (48), with permission from J. Med. Chem.

In addition to 3D and 4D NOE experiments, several important multi-dimensional NMR experiments have recently been developed to identify through-bond correlations (56-60). These experiments are extremely useful for making the ^{1}H, ^{13}C, and ^{15}N assignments of the protein backbone (60). They do not rely on NOEs which can be difficult to interpret, but utilize through-bond connectivities via heteronuclear couplings. In order to assign the amino acid side chains, new experiments have been developed in which through-bond connectivities are established through large one-bond ^{1}H-^{3}C and ^{13}C-^{13}C couplings (56-59), instead of the conventional manner by correlating the protons via small, conformational dependent ^{1}H-^{1}H three-bond couplings.

Isotope Labeling Techniques - In order to obtain the sensitivity required to apply many of the heteronuclear multi-dimensional NMR experiments, proteins must be isotopically labeled. Perhaps the most useful are the uniformly ^{15}N- and ^{13}C-labeled proteins. Most of the assignments and structural constraints can be obtained using double-labeled proteins. Bacterially expressed proteins can be uniformly ^{15}N- and ^{13}C-labeled using ^{13}C-/^{15}N-labeled media prepared from algal or bacterial extracts (68). Alternatively, proteins can be uniformly ^{13}C- or ^{15}N-labeled by growing bacteria in minimal media containing [U-^{13}C]glucose (or [U-^{13}C]acetate) (69) or ^{15}N-labeled ammonium chloride as the sole carbon or nitrogen source, respectively. Proteins can be selectively labeled by growing bacteria in chemically defined media containing the isotopically-labeled amino acid(s). These proteins can be used in NMR studies to unambiguously assign resonances by amino acid type using isotope-editing techniques (63). Other types of labeling have also been shown to be of value in NMR studies of proteins. Deuterated proteins have been used to simplify spectra (70), produce narrower ^{1}H NMR signals (64), and make stereospecific assignments (71). Non-randomly ^{13}C-labeled proteins have been employed to stereospecifically assign valine and leucine methyl groups (65).

Conformation and active site environment of bound ligands - A variety of approaches have emerged for studying large molecular complexes by NMR (48,72). One of the best approaches for studying receptor-bound ligands involves the use of isotope-editing techniques in which the proton signals of an isotopically labeled ligand are selectively observed in the presence of the many signals of its target site (49-54). Using this approach both the conformation of a bound ligand and those portions of the ligand that bind to the target site can be determined. These experiments can be conducted using two-dimensional isotope-edited NMR methods (49-52). Alternatively, for futher simplifying the spectra in cases when the proton NMR signals of the ligand overlap, heteronuclear three-dimensional NMR experiments may be employed in which the remaining proton NMR signals of the ligand are edited in a third dimension by their ^{13}C or ^{15}N frequencies (53,54). The advantages of these techniques is that important structural information for designing new analogs can be obtained on new drug/receptor complexes in as little as 2-3 weeks. Furthermore, the receptor protein does not have to be very pure, since the isotopically-labeled ligand binds selectively to the protein of interest and only the signals of the ligand and the nearby protein are detected.

An example of the type of structural information that can be obtained by NMR on an isotopically-labeled drug molecule bound to its receptor is illustrated by NMR studies on the cyclosporin A/cyclophilin complex. Cyclosporin A (CsA), **25**, a clinically useful immunosuppressant, exerts its activity through its interaction with cyclophilin (CyP), a 17.8 kD protein (165 residues) (73,74). In order to aid in the design of more potent and potentially less toxic CsA analogs, NMR studies of the CsA/CyP complex were initiated to determine the bound conformation of CsA and to identify those portions of CsA that bind to CyP. From an analysis of a ^{13}C-resolved 3D NOE spectrum of [U-^{13}C] CsA bound to CyP, three-dimensional structures of CsA (Fig. 7) were calculated using a distance geometry/dynamical simulated annealing protocol in which proton-proton distances calculated from the 3D NOE data were included as constraints (53). As shown in Figure 8, the conformation of CsA when bound to cyclophilin as determined by NMR was found (51-53) to be very different from the conformation of uncomplexed CsA determined by X-ray crystallography and NMR spectroscopy (75).



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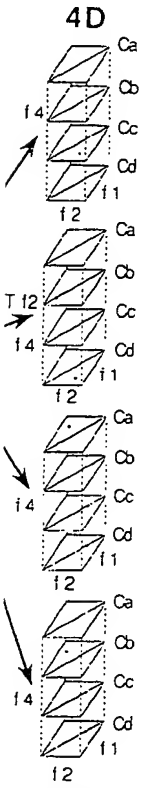

and 4D NOE
l, and Hc/Hd.
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Figure 7. Superposition of the 20 CsA structures with the lowest NOE energy contribution out of 95 converged structures. From (53), with permission from Biochemistry.

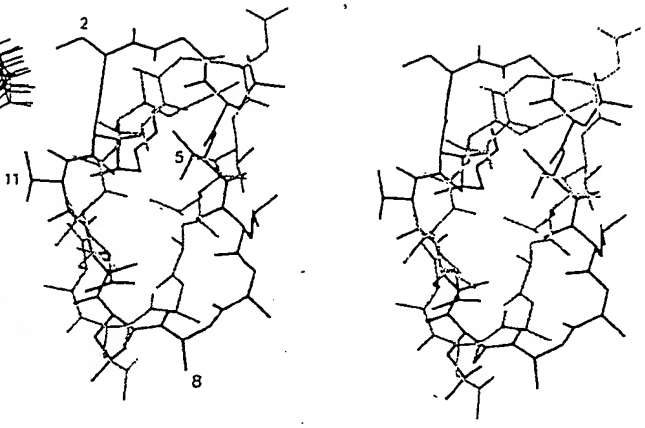

Figure 8. Stereoview of CsA (restrained energy minimized average structure generated from 95 individual structures) bound to cyclophilin (black) superimposed on the X-ray crystal structure of CsA (grey) (75) determined in the absence of cyclophilin. From (53), with permission from Biochemistry.

In addition to the conformation of CsA when bound to cyclophilin, the NMR studies also revealed those portions of CsA that interact with cyclophilin from CsA/CyP NOEs (53,76). As shown in Figure 9, the protons attached to the filled and checkered carbon atoms were found to be in close proximity to cyclophilin as evidenced by NOEs between these CsA protons and the protein. These data suggest that CsA residues 1, 2, 9, 10, and 11 are involved in binding to cyclophilin and are consistent with the structure/activity relationships (77,78) that indicate the importance of these CsA residues for cyclophilin binding and immunosuppressant activity. Those portions of CsA that bind to CyP and those that are exposed to solvent were also distinguished by measuring the proton T_1 values of ^{13}C-labeled CsA in the presence and absence of a paramagnetic relaxation reagent, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO) (79). Large effects of the spin label were observed for the protons attached to the jagged and checkered filled carbon atoms (Fig. 9), indicating that CsA residues 4, 6, 7,8 are exposed to solvent. These results are consistent with the structure/activity relationships of CsA analogs in which cyclophilin binding was found to be relatively insensitive to modifications at these sites (77,78).

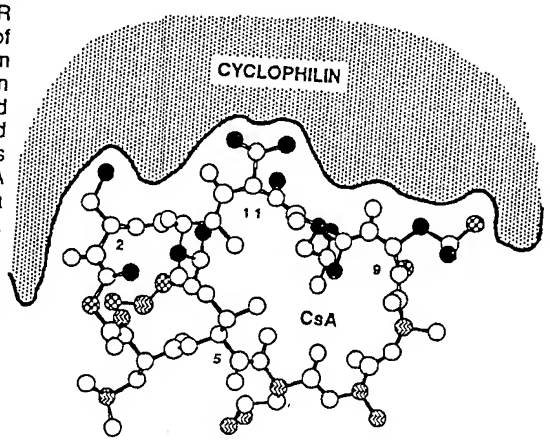

Figure 9. Three-dimensional structure of CsA bound to cyclophilin. CsA/cyclophilin NOEs were observed from CsA protons attached to the filled and checkered carbon atoms (53). The protons attached to the jagged and checkered carbon atoms exhibited the largest change in relaxation rate upon the addition of HyTEMPO. From (79), with permission from J. Am. Chem. Soc.

The NMR studies described thus far were performed with cyclophilin A. Additional cyclophilins have been identified which could also play an important role in the immune response. One of these, cyclophilin B (CyPB), contains a hydrophobic N-terminal signal sequence and is 64% identical to CyPA (80). From the similar ^1H and ^{13}C chemical shifts and NOE data of [U-^{13}C]CsA bound to CyPB and CyPA, it was concluded that the conformation and active site environment of CsA in the two complexes are nearly identical, except in the vicinity of the MeVal^{11} CsA residue

(81). This approach was extremely useful for identifying subtle structural differences between two drug/receptor complexes in only a few days and could prove useful for designing analogs that bind selectively to only one of the proteins. For example, on the basis of the NMR studies of the CsA/CyPB complex, selectivity might be achieved with a CsA analog modified at the 11-position.

Macromolecular Structure Determination - The general approach for determining complete three-dimensional structures by NMR involves the following steps: i) assigning the NMR signals, ii) obtaining ^{1}H-^{1}H distance constraints from NOE data, iii) obtaining dihedral angle constraints from coupling constants, and iv) calculating structures using distance geometry and restrained molecular dynamics (67,82). Steps (i) and (ii) are greatly facilitated using 3D and 4D NMR experiments. Not only do these experiments resolve the data, thereby simplifying the analysis, but they also allow assignments of the ^{15}N and ^{13}C chemical shifts. In addition to these experiments, new methods have also been devised (83-86) for measuring ^{1}H-^{1}H, ^{1}H-^{15}N, and ^{1}H-^{13}C three-bond J-couplings from 2D and 3D NMR spectra. From these J-values, the stereospecific assignments of Cβ methylene protons can be determined and dihedral angle constraints can be obtained which improve the quality of the calculated structures (87,88).

Currently, the largest protein structure determined by NMR is interleukin-1β (IL-1β) (66). Conventional 2D NMR methods were largely unsuccessful for determining the structure of this 153 residue protein due to its relatively large size. However, a variety of heteronuclear double- and triple-resonance 3D NMR experiments resulted in the assignment of a complete set of ^{1}H, ^{13}C, and ^{15}N resonances, the determination of the secondary structure, the location of bound water molecules, and the investigation of backbone dynamics (66 and references therein). Initial low-resolution structures of IL-1β were obtained using only 446 NOEs obtained from ^{15}N-resolved 3D NOE experiments. The accuracy and precision of these structures was dramatically improved using a much larger number of distance constraints derived from heteronuclear 4D NOE experiments (66). The final structure calculations were done using 2780 distances and 366 torsion angles for a total of 3,146 experimental constraints. With this many constraints, the 3D structure of IL-1β was well-defined (Fig. 10). The atomic rms distribution about the average coordinate positions of the calculated structures was 0.41 +/- 0.04 Å for the backbone atoms and 0.82 +/- 0.04 Å for all atoms except those belonging to residues 1, 152, and 153. From the high resolution structure of IL-1β determined by NMR, the biological activity and receptor binding displayed by chemically modified and mutant proteins could be interpreted. Three distinct binding sites for the IL-1 receptor involving surface residues of IL-1β were postulated (66).

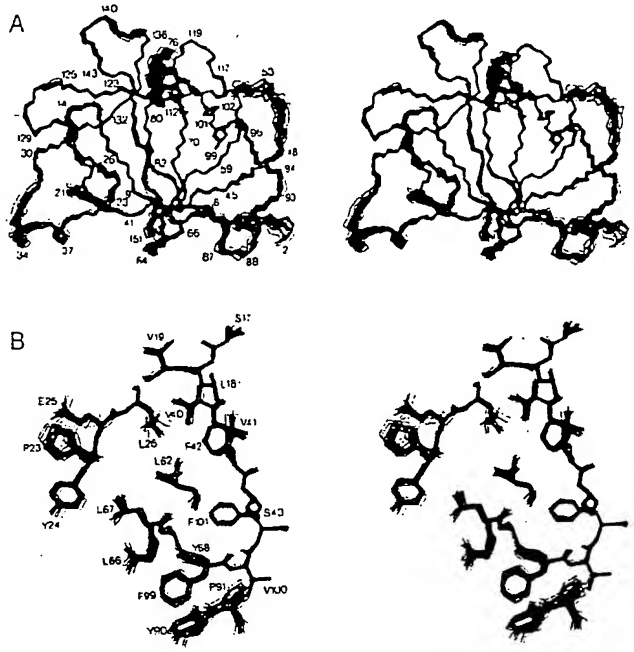

Figure 10. Stereoviews showing the best fit superposition of the (A) backbone atoms of IL-1β and (B) all heavy atoms of a selected region of IL-1β. Adapted from (66), with permission from Biochemistry.

Limitations and future developments - Currently, NMR can be practically applied to the study of macromolecules with molecular weights less than about 40 kDa. This limitation is mainly due to the broad NMR signals for molecules of this size and the corresponding loss in signal intensity observed

in multi-dimensional NMR experiments. A second limitation is the requirement that the protein or molecular complex be soluble to at least 1 mM. For certain studies, a third limitation is the requirement for isotopically-labeled ligands, particularly for those studies aimed at determining the complete high resolution structure of a protein larger than 100 residues. This requires that the protein can be expressed in active form at relatively high levels (1 mg/ml) in a bacterial expression system. Ideally, both labeled proteins and labeled receptors would be available for NMR studies. Initial studies should involve labeled ligands bound to unlabeled receptors to determine the bound conformation of the ligand and to identify those portions of the ligand that interact with the receptor. Alternatively, unlabeled ligands could be studied when bound to labeled proteins using recently developed NMR techniques (89) with the goal of determining the bound structure of the ligand and its active-site environment. The next step would involve the complete three-dimensional structure determination of the drug/receptor complex using isotopically labeled receptor proteins and multi-dimensional NMR techniques. Once an initial structure is determined, additional three-dimensional structures of the same receptor bound to different ligands could be rapidly obtained, facilitating the design of new analogs in an interactive manner.

Future developments are expected to improve the speed and accuracy in which three-dimensional structures can be determined by NMR. The accuracy of the structures, important for designing analogs with a precision required for tight binding to the receptor, may be improved by more accurately interpreting the NOE data and by obtaining more constraints. Three-dimensional structures may be obtained more rapidly using new heteronuclear multi-dimensional NMR experiments and improved software tools, reducing the time required both for signal assignment and extraction of the relevant structural parameters used in calculating the structures.

COMPARISON OF X-RAY CRYSTALLOGRAPHY AND NMR

NMR Solution and X-Ray Crystal Structures: Similarities and Differences - Comparisons of protein structures determined using both NMR and X-ray crystallography have shown that the solution and crystal structures of a protein are in general agreement overall (90-92). This is not surprising since protein crystals are highly hydrated (27-65% solvent content) and can be treated as well ordered, highly concentrated protein solutions. Well-determined NMR and X-ray crystal structures usually do not deviate by more than about 1 Å rms for backbone atoms (90-92). The degree of structural correspondence between X-ray and NMR structures is greatest for residues in well-ordered secondary structural elements and in the interior, or core, of a protein, and is least for surface residues. It should be noted that different crystal forms of the same protein may deviate by 0.4-0.5 Å rms for backbone atoms. These differences, which are also most pronounced at the protein surface, are commonly attributed to the effects of different crystal packing arrangements as well as experimental errors.

Protein crystals are formed by a regular, three-dimensional network of protein-protein interactions stabilized by the high protein concentrations (typically on the order of 10 mM) within crystalline environments. These environments may induce order-disorder transitions for localized regions of a structure, stabilize flexible side chains or polypeptide segments that may assume various conformations in dilute solutions, as well as influence quaternary structure. Thus, crystal packing forces may influence a segment of a protein to assume a conformation not ordinarily found in dilute solution. In contrast, NMR structures are unbiased by crystal packing forces and can give a more accurate picture of the relative mobilities of side chains located at a protein's surface. Moreover, the extent of crystal packing influences on protein structures can be revealed by NMR studies. These influences may be localized to individual side chains, or may extend over secondary structural elements. For example, in the crystal structure of C3a (93), a 77 residue complement protein, both the N- and C-terminal regions make intermolecular crystal contacts. The crystal structure of the protein contains a long C-terminal helix and a disordered N-terminus. NMR studies of C3a (94) clearly indicated that the C-terminal hexapeptide was in a random coil conformation, while the N-terminus exhibited a helix in a region that was disordered in the X-ray crystal structure. The C-terminus of C3a contains the receptor recognition site, and an accurate conformation for this region is critical for the structure-based drug design of C3a antagonists.

In an example of possible crystal packing effects on subunit-subunit interactions, comparison of the X-ray (92) and NMR (95) structures of interleukin-8 (IL-8) revealed that this dimeric immunoregulatory protein can undergo a rearrangement of quaternary structure that controls the size of the cleft formed by the separation of two, C_2-related α-helices from individual monomers. In addition, the crystal and solution structures exhibited strikingly different hydrogen-bonding patterns

for His33, which accepts a hydrogen bond from a backbone amide of Gln8 in the solution structure, but donates a hydrogen bond to the backbone carbonyl of Glu29 in the crystal structure.

The conformation of a small molecule ligand when bound to its receptor in solution may differ dramatically from the conformation exhibited in the uncomplexed form either in solution or in the crystalline state. Thus, small molecule X-ray crystal or NMR structures must be regarded cautiously, and one should not assume that these structures are representative of the bioactive conformation. As an illustration of this point, NMR solution studies demonstrated that the conformation of CsA when complexed to CyP differed substantially from the crystal structure of CsA (51-53). Similarly, X-ray crystallographic studies of the structure of the immunosuppressant, FK-506, complexed with its receptor, FK-506 binding protein (FKBP), indicated that FK-506 exhibited dramatically different conformations in the bound and free crystalline states (96). These results were confirmed by NMR studies on the solution structure of an FK-506 analogue, ascomycin, complexed with FKBP (54). The solution structure of FK-506 in chloroform was also determined by NMR (97) and found to be conformationally dissimilar to both the uncomplexed and bound crystal forms.

X-Ray Crystallography and NMR as Complementary Techniques - Structural information obtained by X-ray crystallography can aid in the structure determination of the same or of a homologous system by NMR spectroscopy and *vice versa*. For example, once the structure of a receptor is known from X-ray studies, this information can be used to aid the interpretation of NOE signals from isotopically-labeled ligand/receptor complexes, and thereby facilitate the structural analysis of ligand/receptor interactions in solution. This strategy was employed in the determination of the bound conformation of a renin inhibitor complexed with pepsin (49). Knowledge of the crystal structure of a related inhibitor/pepsin complex aided in assigning enzyme-ligand cross peaks, and resulted in a model for the docked inhibitor. This strategy is particularly important for cases where crystals of the relevant complex do not exist. In a more recent example (98), the NMR solution structure of the bound conformation of CsA (52) was docked with the X-ray crystal structure of native CyP (99). The complex was refined by molecular dynamics using the crystal structure of CyP and the intermolecular NOEs as constraints. Comparison of the resultant model structure with a crystal structure of a tetrapeptide/CyP complex provided experimental evidence to support the hypothesis that the MeBmt[1] carbinol group of CsA mimics the transition state or intermediate structure in the PPIase reaction (100).

NMR can also provide valuable information for the X-ray crystallographic structure determination of proteins. The secondary structure of a protein in solution can be accurately and quickly determined by NMR studies and used to help fit a polypeptide backbone into an electron density map. NMR structural data were used in this manner to assist in the electron density map interpretations for the recent three-dimensional X-ray crystal structure determinations of CyP (101) and the FK-506/FKBP complex (96). In addition, three-dimensional protein structures determined by NMR could be used as starting models for molecular replacement phasing. This was shown in test experiments using the known crystal structures of crambin (102) and tendamistat (103). Recently, the NMR solution structure of IL-8 was used in a molecular replacement study to solve the unknown crystal structure of IL-8 at 1.8 Å resolution (92). Previous attempts to solve IL-8 using the crystal structure of a closely related protein, platelet factor 4, gave unsatisfactory results, and attempts to prepare suitable heavy atom derivatives were unsuccessful. Thus, the availability of the NMR structure of IL-8 was critical to the successful crystal structure determination for this immunoregulatory factor. This experience clearly demonstrates the high degree of structural accuracy that NMR is capable of providing, and it is expected that there will be a growing demand for NMR-derived structures to assist in X-ray crystallographic studies.

FUTURE PROSPECTS

The promise of using three dimensional atomic structures of receptors to aid medicinal chemists in their drug design efforts is coming of age. Concomitant advances in numerous fields are converging to make the interdisciplinary approach of structure-based drug design applicable to an ever-growing number of important targets. As a recent example, the AIDS epidemic has resulted in the most intensive search for an antiviral agent ever witnessed in spite of the remarkable fact that no viral disease has ever been completely cured or controlled by chemotherapy. The reassessment of our approach to antivirals that is behind this effort is largely due to targeted and structure-based strategies to anti-HIV drug design that have become possible owing to a number of rapid scientific advances. In the past four years, the structures of HIV-1 protease (104-106), HIV-1 RNase H (107) and the HIV gp120-binding domain of the CD4 receptor (109,109) have been determined using X-

ray crystallographic methods, and the solution structure of the HIV-1 p7 nucleocapsid protein has been determined by NMR (110). This short litany of HIV-related structural studies illustrates the power that modern molecular and structural biology approaches can bring to drug discovery programs in almost any area.

Although structural protein or enzymes are primary targets for drug design, protein/nucleic acid complexes and hormone/receptor complexes comprise two other classes of therapeutically important targets. No structures of medically relevant protein/nucleic acid complexes have been reported thus far, but the growing number of structures of regulatory proteins complexed with oligodeoxyribonucleotides (111) indicates that they are certainly feasible targets for structure-based design. Recently, the crystal structure of human growth hormone (hGH) complexed to the recombinant soluble extracellular domain of its receptor was determined by X-ray crystallography (112). Besides providing a detailed picture of the interactions between the polypeptide ligand and its receptor, this structure provides new insights into the structural basis of signal transduction which may be mediated through hormone-induced dimerization of receptor molecules. This study was possible owing to the soluble nature of the hormone-binding domain of the hGH receptor. Many receptors are relatively insoluble, integral membrane-bound proteins whose structure elucidation by either X-ray crystallography or NMR is much more difficult owing to the technical problems of achieving large, well-ordered three dimensional crystals and the broad linewidths of the NMR signals expected for these molecules in solution. However, if these obstacles can be surmounted, the structure determination of this important and abundant class of targets would become accessible. Advances in crystallization of membrane proteins (113) has led to the X-ray structure determination of a bacterial photosynthetic reaction center (114) and a trimeric pore-forming protein, porin, from a bacterial outer membrane (115). Two dimensional crystals, or well-ordered sheets, of membrane proteins are often easier to obtain than three-dimensional crystals. In these cases, high resolution electron microscopy can provide valuable structural information. Recently, this technique was employed to determine the 3.0 Å structure of bacteriorhodopsin from a purple membrane (116). High resolution electron microscopy promises to become a more widely used method particularly for determining structural data on well-ordered aggregates of membrane proteins and other macromolecular assemblies.

The limits of the structure-based drug design approach have not yet been realized, and many of the difficulties in successfully applying this method are largely theoretical. Major conceptual challenges lie ahead in the areas of structure and binding affinity prediction. While methods are being developed in these areas, experimental structure determination efforts using X-ray crystallography and NMR will continue to provide critical information that can be used to design more effective drugs. The ultimate success of the structure-based drug design approach will depend, in any case, on the wisdom of target selection and on the ability to marry together teams of scientists that contain the requisite scientific and collaborative skills.

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ANNUAL REPORTS IN MEDICINAL CHEMISTRY
Volume 27

Sponsored by the Division of Medicinal Chemistry of the American Chemical Society

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ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Boston London Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Academic Press, Inc.
1250 Sixth Avenue, San Diego, California 92101-4311

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Catalog Number: 66-26843

International Standard Book Number: 0-12-040527-X

PRINTED IN THE UNITED STATES OF AMERICA
92 93 94 95 96 97 QW 9 8 7 6 5 4 3 2 1

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